

Autonomous Microbial Genosensor

David Fries and John Paul

U. South Florida

Marine Science Dept

140 7TH Ave So.

St. Petersburg, FL 33701

Phone (727) 553-3961 Fax (727) 553-3967 Email: dfries@marine.usf.edu

Phone (727) 553-1168 Fax (727) 553-3967 Email: jpaul@usf.edu

Grant #: N00014-02-1-0265

LONG-TERM GOALS

The overall goal of this work involves the development of a self contained, fieldable microfluidic based biochemical sensor for detecting microbial water quality indicators. The targeted integrated system comprises an opto-analytical segment, integrated detection electronics, and a fluidic management system. The long term goal is to place the autoanalyzer onto field platforms.

OBJECTIVES

The main focus of the effort is to advance the design, fabrication and field testing of a microfluidic device as biochemical analyzer for detecting microbial water quality indicators. The goal is to develop an autoanalyzer that can be adapted to engineered gene probes for identification of target microorganisms in the field and has applications toward harmful algal blooms, water-borne pathogens and biowarfare agents and also environmental monitoring.

APPROACH

The current period entails several main objectives. Distinguishable differences between automated and standard bench-top NASBA protocols are being identified. Integration of the sample preparation and detection into a linear tape drive mechanism has shifted into the more pragmatic solution of a modular wheel system. Modules for sample filtration and lysis, mRNA purification, and amplification and detection are being constructed to meet the fluidic requirements of automated NASBA protocols. Accordingly, fluidic management is being optimized to accommodate this very dynamic system. Additional peripheral components for power supply and wireless data transfer are also being integrated.

WORK COMPLETED

RNA extraction and purification

We have developed a protocol to capture and chemically lysis *Synechococcus* spp. on a 0.45µl durapore filter. The lysis protocol utilizes enzymatic digestion followed by treatment with a chaotrophic salt. NASBA amplification using the passive lysis method has been shown to produce equivalent or better results than mechanical disruption methods (e.g. bead beating).

Report Documentation Page				Form Approved OMB No. 0704-0188	
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
1. REPORT DATE 30 SEP 2003		2. REPORT TYPE		3. DATES COVERED 00-00-2003 to 00-00-2003	
4. TITLE AND SUBTITLE Autonomous Microbial Genosensor				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) U. South Florida,,Marine Science Dept,140 7TH Ave So,,St. Petersburg,FL, 33701				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The overall goal of this work involves the development of a self contained, fieldable microfluidic based biochemical sensor for detecting microbial water quality indicators. The targeted integrated system comprises an opto-analytical segment, integrated detection electronics, and a fluidic management system. The long term goal is to place the autoanalyzer onto field platforms.					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Same as Report (SAR)	18. NUMBER OF PAGES 6	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

RNA purification protocols based on the Boom RNA extraction method are currently being assessed. Two prototype RNA purification columns have been designed and built in-house. Based around standard micropipette tips and solid phase silica matrix, the designs have been assessed for their ability to adequately remove the contaminating ethanol wash buffer using a two-pump system in push/pull fashion. One prototype showed promising results and has led to the design of a purpose built RNA extraction column. Future work is focusing on a chemical method to further remove the ethanol buffer from these columns.

Real-Time NASBA

Using the Nuclisens[®] basic kit (bioMérieux, Boxtel NL), a real-time NASBA assay has been developed to detect as little as ~0.01 fg of *in vitro* transcribed *rbcL* RNA. When applied to cultured cells the assay can detect 10-100 cells of *Synechococcus* sp. WH7803. The specificity of the assay has been tested against a range of target and non-target organisms, and has been shown to be specific to only *Synechococcus* spp. containing Form 1A RubisCO. Coupled with the on filter chemical lysis protocol and RNA purification through commercially available RNA extraction columns, the real-time NASBA assay has been detected *Synechococcus* spp. from sea water samples obtained from Bayboro Harbor, St. Petersburg, FL. (Figure 1)

Single shot real-time NASBA

We have successfully developed one-reaction liophilized spheres containing all the reagents required to perform a real-time NASBA. The “all in one” spheres contain primers, molecular beacons and the trimeric enzyme cocktail thereby reducing the fluid handling requirements of the Autoanalyzer. Similarly the presence of the enzymes at the initial reaction mix ensures a cold start for the reaction, thereby simplifying the heating logic requirements of the analyzer. Initial results using *in vitro* transcribed RNA indicates no loss assay fidelity of reactions undergoing hot or cold starts.

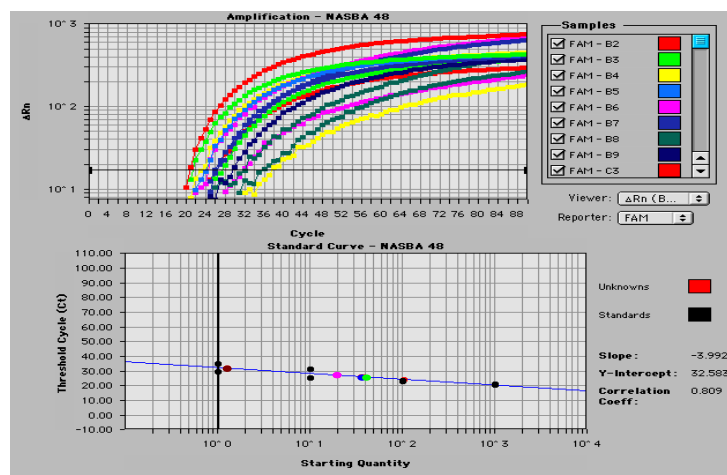


Figure 1. Real-time NASBA amplification and detection of *Synechococcus rbcL*. Real time amplification plot and standard curve of a real-time NASBA containing cultured *Synechococcus* sp. WH7803 cells and seawater, the standard curve was constructed using known quantities of *in vitro* transcript (black points). Key: ● - 1000 cells of cultured WH7803, ● - 100 cells of cultured WH7803, ● - seawater containing ~2600 cells ● - seawater containing ~700 cells, ● - seawater containing ~200 cells.

Instrumentation Development

The heat integrated fluorometer components have been optimized and tested. (Figures 2 and 3) The reaction cell has been modified from a flow through system into a wheel system, which accommodates 25 200ul PCR tubes that index through the optical block. Figure 4. One tube per reaction.

Additionally, thermal regulation of the tube from the Ion Optics T-05 infrared heater is monitored by a Malexis thermopile, a calibrated temperature sensor focused with an infrared lens. Indexing of the reaction wheel by a stepper motor is controlled by the same microcontroller, which controls all system functions from a newly designed and fabricated mainboard. This custom printed circuit board incorporates the anticipated expansion capabilities necessary to complete all modules to the system. Data transfer is being accomplished using an Xport wireless web server / RS232-10base-T converter. The entire 24volt system is powered by a rechargeable lithium-ion battery pack. (Figures 4 and 5)

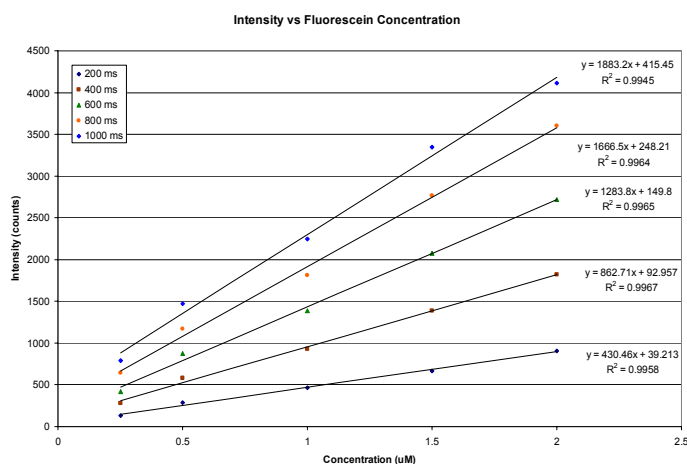


Figure 2. Plots of light intensity counts vs fluorescein concentration

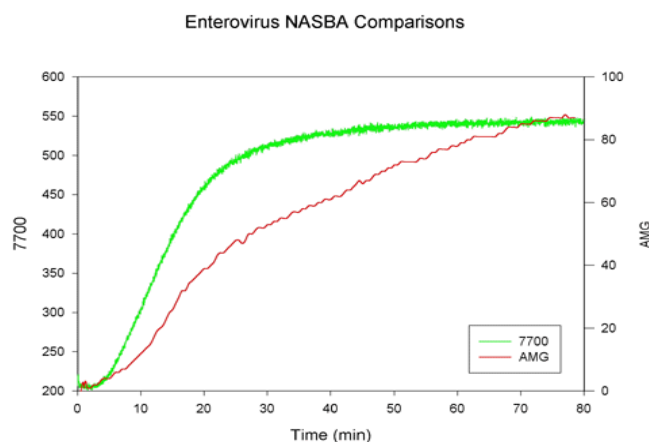


Figure 3. Real time NASBA comparisons

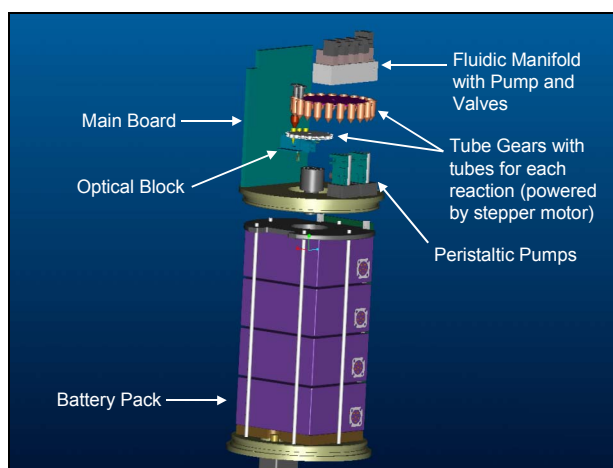


Figure 4. Illustrative model of current system configuration

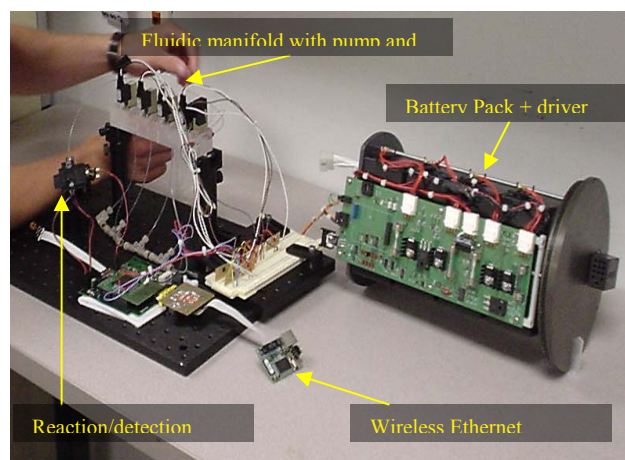


Figure 5. Photograph of actual components

A Tuthill positive displacement pump is being tested to push the whole water sample through a .45 micron filter. Peristaltic and diaphragm pumps are being combined in a push pull fashion to allow for

proper buffer and reagent dispensing from the custom Burkert 6603 3-way valve manifold. (Figure 6) As seen in Figure 7, microfluidic control can be controlled by varying pump speeds and adjusting valve timing. Additional manifold prototypes have been developed to interface fluidics with appropriate wheel components.

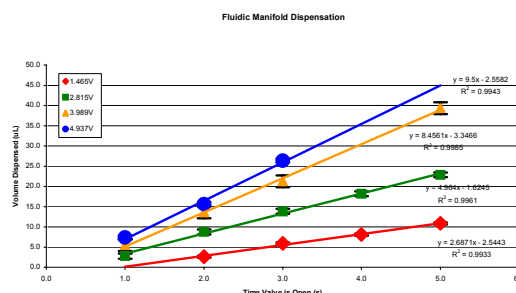
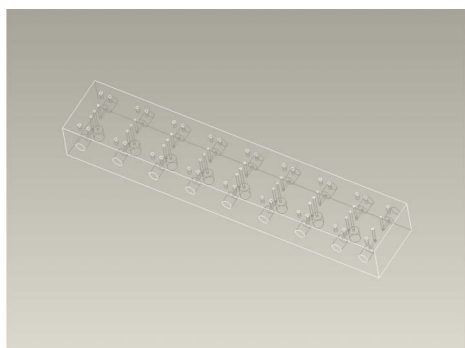


Figure 6. 3-D drawing of custom manifold **Figure 7. Plots of fluid dispensation vs valve timing**

RESULTS

We have advanced both molecular diagnostic technology and instrumentation toward the goal of a remote, deployable microbial system for field genomics. We have demonstrated NASBA amplification of the intended environmental target (*synechecoccus*) and have made several of the key instrumentation modules (fluorometer, heater, controller) needed for the microfluidic-based autonomous analyzer system.

IMPACT/APPLICATIONS

This proof of technology demonstration has impact for both marine science and ocean systems applications that seek real-time detection and characterization of microorganisms for water security, modeling the biota, and seeking the detection or discrimination of species that may indicate the presence of man-made or natural changes in a targeted region.

TRANSITIONS

The work described herein on the microbial sensor development is an emerging ocean detection capability. We expect this technology to have application beyond coastal ocean science into freshwater and drinking water security, and as an key detection component in the emerging global ocean observing efforts that seek real-time detection biosensors.

RELATED PROJECTS

We are involved in another (NSF) project aimed at a field sensor for water monitoring applications to detail ecosystems biocomplexity.

PATENTS, PUBLICATIONS, TECHNOLOGY TRANSFER

Development of an Autonomous Microbial Genosensor (AMG), Matthew C. Smith, John H. Paul, David P. Fries & Andrew Farmer, Marine Biotechnology Summit, Ft Pierce. FL. Oct. 2002, accepted

Developing NASBA for Real-Time Detection of Microbial RNA Targets in the Marine Environment, Matthew C. Smith, John H. Paul, David P. Fries, ASLO, Salt Lake City, UT. Feb. 2003, oral, submitted

Autonomous Genosensor, D.Fries, A.Farmer, J. Paul, M. Smith, Patent Submitted Mar, 2003